Fibroblast Growth Factor 21 Prevents Atherosclerosis by Suppression of Hepatic Sterol Regulatory Element-Binding Protein-2 and Induction of Adiponectin in Mice

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Background—Fibroblast growth factor 21 (FGF21) is a metabolic hormone with pleiotropic effects on glucose and lipid metabolism and insulin sensitivity. It acts as a key downstream target of both peroxisome proliferator-activated receptor α and γ, the agonists of which have been used for lipid lowering and insulin sensitization, respectively. However, the role of FGF21 in the cardiovascular system remains elusive.

Methods and Results—The roles of FGF21 in atherosclerosis were investigated by evaluating the impact of FGF21 deficiency and replenishment with recombinant FGF21 in apolipoprotein E−/− mice. FGF21 deficiency causes a marked exacerbation of atherosclerotic plaque formation and premature death in apolipoprotein E−/− mice, which is accompanied by hypoadiponectinemia and severe hypercholesterolemia. Replenishment of FGF21 protects against atherosclerosis in apolipoprotein E−/− mice via 2 independent mechanisms, inducing the adipocyte production of adiponectin, which in turn acts on the blood vessels to inhibit neointima formation and macrophage inflammation, and suppressing the hepatic expression of the transcription factor sterol regulatory element-binding protein-2, thereby leading to reduced cholesterol synthesis and attenuation of hypercholesterolemia. Chronic treatment with adiponectin partially reverses atherosclerosis without obvious effects on hypercholesterolemia in FGF21-deficient apolipoprotein E−/− mice. By contrast, the cholesterol-lowering effects of FGF21 are abrogated by hepatic expression of sterol regulatory element-binding protein-2.

Conclusions—FGF21 protects against atherosclerosis via fine tuning the multiorgan crosstalk among liver, adipose tissue, and blood vessels. (Circulation. 2015;131:1861-1871. DOI: 10.1161/CIRCULATIONAHA.115.015308.)

Key Words: adipokines ■ atherosclerosis ■ fibroblast growth factor 21 ■ hormones

Fibroblast growth factor (FGF) 21 is a member of the endocrine FGF subfamily that is produced predominantly in the liver. Physiologically, FGF21 plays a key role in mediating the metabolic responses to fasting/starvation by enhancing fatty acid oxidation and ketogenesis and inducing growth hormone resistance. Pharmacologically, therapeutic intervention with recombinant FGF21 has been shown to counteract obesity and its related metabolic disorders in both rodents and nonhuman primates, including reduction of adiposity and amelioration of hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, and fatty liver disease. Furthermore, FGF21 is the downstream target of both peroxisome proliferator-activated receptor (PPAR) α and PPARγ, and a growing body of evidence suggest that the glucose-lowering and insulin-sensitizing effects of the PPARγ agonists (thiazolidinediones) and the therapeutic benefits of the PPARα agonists (fenofibrate) on lipid profiles are mediated in part by induction of FGF21.

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FGF21 exerts its metabolic actions by binding to the complex receptor between the FGF receptor (FGFR) and β-klotho, a single transmembrane protein that is highly expressed in adipose tissue, liver, pancreas, and hypothalamus. Adipocytes are the primary target of FGF21, where it increases glucose.
uptake, modulates lipolysis, enhances mitochondrial oxidative capacity, enhances PPARγ activity, and promotes browning of white adipose tissue. Furthermore, therapeutic administration of FGF21 has been shown to increase the production of adiponectin, an adipocyte-secreted hormone with insulin-sensitizing, anti-inflammatory, and vascular protective activity. Adiponectin knockout mice are resistant to the effects of FGF21 on alleviation of insulin resistance, hyperglycemia, dyslipidemia, and fatty liver disease associated with dietary or genetic obesity, suggesting that adiponectin acts as an obligatory downstream mediator of FGF21 on energy metabolism and insulin sensitivity. In addition, FGF21 has also been shown to exert its direct actions on the pancreas, hypothalamus, heart, and liver, acting as a mediator to coordinate the multiorganic crosstalk under various pathophysiological conditions.

Although the metabolic functions of FGF21 are well characterized, little is known about its pathophysiological roles in atherosclerosis, a chronic inflammatory disease intimately associated with metabolic syndrome. A number of clinical studies have observed an increased circulating level of FGF21 in patients with atherosclerosis or those individuals who are at high risk of developing this disease. In both rhesus monkeys and humans with obesity and diabetes mellitus, chronic administration of FGF21 decreases low-density lipoprotein (LDL) cholesterol and increases high-density lipoprotein cholesterol. However, whether such a beneficial effect of FGF21 on lipid profiles is sufficient to render a protection against atherosclerotic diseases has not been explored. To address this issue, we investigated the impact of both FGF21 deficiency and replenishment on the pathogenesis of atherosclerosis in apolipoprotein (apo) E−/− mice. Our results showed a markedly aggravated atherosclerotic phenotype of FGF21 knockout mice, which can be reversed by replenishment of FGF21. Therefore, we further investigated the mechanisms whereby FGF21 protects atherosclerosis via its multiple actions in both adipose tissue and liver.

Methods

Additional details of mice and experimental procedures are included in the online-only Data Supplement. All of the animal studies were approved by the animal research ethics committees of Wenzhou Medical University and the University of Hong Kong.

Statistical analysis was performed using either the Mann-Whitney U test or the Kruskal-Wallis test when more than 2 experimental conditions were compared. When the global Kruskal-Wallis test was significant, pairwise comparisons were performed with the Dunn-Sidak procedure for multiple corrections. Repeated-measure ANOVA was used to compare circulating FGF21 levels between wild-type and FGF21−/− mice at different time points, as well as serum levels of FGF21 and adiponectin in FGF21 and apoE−/− double deficiency (DKO) mice at different time points after administration with FGF21 or adiponectin. The survival of mice was compared using Kaplan-Meier survival analysis with a log-rank test. All of the statistical analyses were performed with IBM SPSS version 20.0 (IBM Corporation, Armonk, NY). A value of \( P < 0.05 \) was considered statistically significant.

Results

FGF21 Deficiency Accelerates Atherosclerotic Plaque Formation in ApoE−/− Mice

Several clinical studies have observed a significantly elevated serum level of FGF21 in patients with atherosclerosis. Consistently, both circulating levels of FGF21 and its hepatic mRNA expression were progressively elevated in apoE−/− mice with spontaneous development of hypercholesterolemia and atherosclerosis (Figure 1 in the online-only Data Supplement). To explore the pathophysiological roles...
of FGF21 in atherosclerosis, we generated DKO mice by backcrossing FGF21 knockout mice into apoE−/− mice in C57BL/J background for more than 10 generations. DKO mice were confirmed by both polymerase chain reaction analysis and Western blot analysis of the liver tissue (Figure II in the online-only Data Supplement). There were no obvious differences in food intake and body weight between apoE−/− mice and DKO mice on standard chow (Figure IIIA in the online-only Data Supplement). However, the atherosclerotic lesion area in DKO, as determined by oil red O staining of the entire aorta, was 1.6-fold and 1.8-fold greater at 24 weeks and 52 weeks than age- and sex-matched apoE−/− mice (Figure 2A). Additional histological evaluation showed that the plaque areas in the aortic sinus and brachiocephalic artery of 24-week-old DKO mice were 2.1-fold and 2.9-fold greater than in apoE−/− mice (Figure 1B and 1C). Likewise, both macrophage infiltration and smooth muscle proliferation in the atherosclerotic lesion area of the aortic sinus in DKO mice were significantly higher than in apoE−/− mice (Figure 1D and 1E). Cholesterol ester contents extracted from the brachiocephalic artery of DKO mice were also much higher than those in apoE−/− mice (Figure 1F), suggesting that FGF21 deficiency renders apoE−/− mice more susceptible to atherosclerosis.

To investigate whether accelerated atherosclerosis in DKO mice decreases longevity, we monitored DKO (n=20) and apoE−/− mice (n=20) on standard chow for 18 months. The surviving rate of DKO was decreased to ±45%, which was significantly lower than that in apoE−/− mice (80%; Figure 1G).

**DKO Mice Display Exacerbated Hyperlipidemia and Augmented Inflammation**

Because FGF21 is an important metabolic regulator, we next investigated whether the atherosclerosis-prone phenotype of DKO mice is attributed to impaired glucose or lipid metabolism. Glucose and insulin levels were comparable between DKO and apoE−/− mice (Figure IIB in the online-only Data Supplement). A glucose tolerance test showed a similar glucose excursion in response to intraperitoneal glucose challenge (Figure IIC and IID in the online-only Data Supplement). On the other hand, DKO mice exhibited a 1.5-fold and 2.1-fold increase in plasma levels of total triglyceride and cholesterol, respectively (Figure 2A and 2B). Additional analysis of lipoprotein compositions demonstrated a significantly increased LDL and very LDL but decreased high-density lipoprotein levels in DKO mice as compared with apoE−/− controls (Figure 2C through 2E).

Quantitative real-time polymerase chain reaction analysis demonstrated a significantly increased expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein-1 (VCAM-1), tumor necrosis factor-α (TNFα), and monocyte chemotactic protein-1 (MCP-1) in aortic tissue, as well as (G–J) plasma levels of these proinflammatory chemokines and cytokines, were measured with real-time polymerase chain reaction and ELISA, respectively. n=6 to 7. Data are presented as dot plots with the line indicating the median. The Mann–Whitney U test was used for comparison of 2 groups.
FGF21 Exerts Its Antiatherosclerotic Effects via Both Adiponectin-Dependent and -Independent Mechanisms

Adipocytes are the primary target of FGF21, where it induces the expression and secretion of adiponectin, an adipokine with insulin-sensitizing, anti-inflammatory, and antiatherosclerotic activities.\textsuperscript{23–25} Because the insulin-sensitizing actions of FGF21 are mediated by adiponectin,\textsuperscript{12,13} we next investigated whether FGF21 exerts its antiatherosclerotic activities via induction of adiponectin. As expected, both circulating levels of adiponectin and its mRNA expression in different adipose depots, including epididymal, subcutaneous, perivascular, and perirenal adipose tissues, were significantly reduced in DKO mice as compared with apoE\textsuperscript{+/-} mice (Figure 3A and 3B). Daily administration of recombinant mouse FGF21 (rmFGF21) for a period of 16 weeks led to higher circulating levels of adiponectin in DKO mice (Figure VA and VB in the online-only Data Supplement), which was accompanied by a significant reduction of atherosclerotic lesion area, as determined by both oil red O staining of the entire aorta and histological quantification of plaque areas between the sinus aorta and brachiocephalic arteries (Figure 3C through 3E).

Chronic administration of recombinant mouse adiponectin (Figure VC in the online-only Data Supplement) also alleviated atherosclerotic plaque formation in DKO mice, whereas the magnitude of reduction in atherosclerosis by adiponectin was significantly smaller than that by rmFGF21.

Further histological analysis demonstrated that rmFGF21 and adiponectin caused a similar degree of decrease in collagen composition, smooth muscle proliferation, and macrophage infiltration (Figure 4A). The magnitude of reduction in expression of proinflammatory chemokines intercellular adhesion molecule-1 and vascular cell adhesion protein-1 and cytokines tumor necrosis factor-\(\alpha\) and monocyte chemotactic protein-1 was also comparable between rmFGF21- and adiponectin-treated DKO mice (Figure 4B through 4F). However, in adiponectin-treated DKO mice, cholesterol ester contents in brachiocephalic arteries were reduced only by 22%, which was significantly lower than rmFGF21-mediated reduction (56%; Figure 4G). Notably, whereas rmFGF21 decreased total cholesterol in DKO mice to a level comparable with apoE\textsuperscript{+/-} mice, adiponectin had no effect on hypercholesterolemia caused by FGF21 deficiency (Figure 4H), despite that both rmFGF21 and adiponectin had a similar potency in decreasing hypertriglyceridemia in DKO mice (Figure 4I). We next compared the direct effects of adiponectin and rmFGF21 in several types of blood vessel cells. Consistent with previous reports,\textsuperscript{26,27} recombinant adiponectin directly inhibited platelet-derived growth factor--induced proliferation and migration of human smooth muscle cells (Figure VIA and VIB in the online-only Data Supplement) and also reduced the uptake of acetylated LDL in peritoneal macrophages (Figure VIC in the online-only Data Supplement). However, rmFGF21 had no direct effect on these cells.

**FGF21 Suppresses Cholesterol Biosynthesis and Enhances Cholesterol Efflux in Mice**

Because our data suggest that the cholesterol-lowering effects of rmFGF21 are independent of adiponectin, we further explored the mechanisms by which FGF21 modulates cholesterol metabolism in mice. The intestinal absorption of cholesterol, as measured by the fecal dual isotope ratio of \textsuperscript{14}C:3\textsuperscript{H} in feces, was comparable between apoE\textsuperscript{+/-} mice and DKO mice and was not affected by treatment with either rmFGF21 or adiponectin (Figure 5A). There was a modest but significant decrease in cholesterol contents in the feces of DKO mice,
and this change was reversed by treatment with rmFGF21 but not adiponectin (Figure 5B). On the other hand, the excretion of bile acids into the feces was not altered by either FGF21 deficiency or replenishment with rmFGF21 (Figure 5C). The de novo biosynthesis of cholesterol in the liver, as measured with the amount of [1-14C]-acetate incorporated into sterols de novo biosynthesis of cholesterol in the liver, was markedly increased by 1.49-fold in DKO mice but was suppressed by treatment with rmFGF21 (Figure 5D). Likewise, hepatic cholesterol accumulation was elevated by FGF21 deficiency but was suppressed by treatment with rmFGF21 (Figure 5E).

We next evaluated the impact of FGF21 on the expression of key genes involved in cholesterol metabolism in the liver. In DKO mice, hepatic expression of 3-hydroxy-3-methylglutaryl-CoA reductase (a rate-limiting enzyme involved in cholesterol synthesis) and several other cholesterologenic genes was significantly elevated when compared with apoE−/− mice, whereas this elevation in DKO mice was inhibited by administration of rmFGF21 but not adiponectin (Figure 6D). On the other hand, the expression levels of key genes involved in bile acid metabolism and secretion, including cholesterol 7-α-monoxygenase, sterol 27-hydroxylase, sterol 12-α-hydroxylase, and small heterodimer partner, were not altered by either FGF21 deficiency or administration (Figure 6C). DKO mice exhibited a modest elevation in the expression of ABC5 and ABCG8 (Figure 6D), the 2 ATP-binding cassette transporters that promote cholesterol secretion.28 The reduced expression of ABCG5 and ABCG8 was reversed by replenishment with rmFGF21 but not adiponectin.

**FGF21 Inhibits Cholesterol Biosynthesis via Suppression of Sterol Regulatory Element-Binding Protein-2**

Cholesterol homeostasis is orchestrated by a number of transcriptional factors, including sterol regulatory element-binding protein (Srebp)-1a, -1c, and -2; liver X receptors; and farnesoid X receptor.29,30 We next investigated whether FGF21 modulates cholesterol metabolism via these transcription factors. There was no obvious difference in either mRNA or protein expression of liver X receptor α, farnesoid X receptor, and Srebp-1 between DKO mice and apoE−/− mice (Figure 7A and 7B). In contrast, DKO mice exhibited a marked elevation in both mRNA and protein expression of Srebp-2, and this
small interfering RNA, an obvious reduction in Srebp-2 expression was observed at 2 days postinfection (data not shown), and its expression continued to decline to a level comparable with apoE−/− mice at days 6 and 12 after adenoviral Srebp-2 small interfering RNA infection (Figure 8A and 8B). Notably, suppression of Srebp-2 expression reversed hypercholesterolemia in DKO mice caused by FGF21 deficiency and concurrently reduced the expression of several cholesterologenic genes, including 3-hydroxy-3-methylglutaryl-CoA reductase, farnesyl diphosphate synthetase, squalene synthase, and 3-hydroxy-3-methylglutaryl-CoA synthetase, which are all well-known downstream targets of Srebp-2 (Figure 8C). Conversely, the effects of rmFGF21 administration on the alleviation of hypercholesterolemia and suppression of cholesterologenic gene expression were abrogated by adenovirus-mediated expression of Srebp-2 (Figure 8D through 8F).

**Suppressive Effects of FGF21 on Cholesterol Biosynthesis Are Mediated by β-Klotho and FGFR2 in the Liver**

FGF21 exerts its actions by binding to FGFR and its coreceptor β-klotho, the latter of which is highly expressed in the liver. To determine whether the regulatory effects of FGF21 on cholesterol homeostasis are attributed to its direct hepatic actions, we generated the β-klotho liver-specific knockout (β-klotho-LKO) mice by intravenous injection of adenovirus-associated virus encoding Cre recombinase into β-klotho-floxed mice (Figure VIIA and VIIB in the online-only Data Supplement). Daily administration of FGF21 significantly decreased high-fat, high-cholesterol diet–induced hypercholesterolemia, which was accompanied by decreased expression of Srebp-2 and several cholesterologenic genes in β-klotho-floxed mice injected with AAV encoding green fluorescent protein as wild-type control, whereas these effects of FGF21 were largely abrogated in β-klotho-LKO mice. By contrast, the stimulatory effects of FGF21 on adiponectin production were comparable between β-klotho-LKO mice and β-klotho-floxed mice, suggesting that hepatic β-klotho mediates the effects of FGF21 on lowering cholesterol but not on elevating adiponectin levels (Figure VIIIC through VIIIH in the online-only Data Supplement). Among 4 major subtypes of FGFRs, FGFR1 plays a key role in mediating the FGF21 actions in adipose tissues. However, hepatic expression levels of FGFR1 were hardly detectable (Figure VIII A in the online-only Data Supplement). Instead, FGFR4 and FGFR2 were abundantly present in the liver, followed by FGFR3. We next explored the role of these FGFRs in mediating the hepatic actions of FGF21 on cholesterol metabolism using adenovirus-mediated knockdown of their expression. Notably, the inhibitory effects of FGF21 on the expression of Srebp-2 and cholesterologenic genes and hypercholesterolemia were significantly compromised in mice with reduced hepatic expression of FGF21 (Figure VIIIIB through VIIIIC in the online-only Data Supplement). By contrast, these FGF21 actions on cholesterol metabolism were little affected by knocking down the expression of the other 3 FGFRs despite >70% knocking down efficiency (data not shown). Taken together, these findings suggest that the regulatory effects of FGF21 on cholesterol homeostasis are mediated at least in part by the FGFR2–β-klotho complex.
Antiatherosclerotic Effect of FGF21

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Discussion

Despite intensive research on metabolic functions of FGF21, its role in the cardiovascular system has scarcely been explored. This study provides novel evidence that FGF21 deficiency causes a marked exacerbation of atherosclerosis and increased mortality of apoE−/− mice, suggesting that FGF21 is a physiological protector against vascular diseases. In this connection, elevated circulating FGF21 levels in patients and rodents with atherosclerosis may represent the defense mechanism of the body to prevent vascular damage. In support of this notion, upregulated FGF21 has been shown to act as a compensatory mechanism to protect against cerulein-induced pancreatitis, endotoxin-induced sepsis, and acetaminophen-induced acute liver injury.

Atherosclerosis is a chronic inflammatory disease involving multiple cell types at various stages of plaque formation, including endothelial cells, lymphocytes, monocytes/macrophages, and smooth muscle cells. Our histological and immunological analysis demonstrated that depletion of FGF21 in apoE−/− mice causes a markedly increased endothelial activation (as determined by expression of endothelial adhesion molecules), augmented macrophage infiltration and foam cell formation, exacerbated smooth muscle cell proliferation, and collagen deposition, all of which can be reversed by the replenishment of exogenous rmFGF21, suggesting that FGF21 is able to inhibit almost every key pathogenic event of atherosclerosis. However, these antiatherosclerotic effects of FGF21 are not attributed to its direct actions on the vascular walls but attributed to the ability of FGF21 in the induction of adiponectin in adipocytes and reduction of cholesterol biosynthesis in the liver. In support of this notion, the expression of β-klotho, an obligatory coreceptor of FGF21, is hardly detectable in any type of blood vessel cells (Z.L. and A.X., unpublished data, 2016), despite its high abundance in adipose tissue and liver.

Recent studies have demonstrated the effects of FGF21 on the elevation of circulating adiponectin in both rodents and humans. Adiponectin, a gene product of FGF21, can stimulate the gene expression, as well as the protein secretion, of adiponectin in adipocytes, macrophages, and smooth muscle cells. Adiponectin possesses potent anti-inflammatory and antiatherosclerotic activities via its multiple actions on blood vessels. In humans, hypoadiponectinemia is an independent risk factor for vascular inflammation and atherosclerosis. In contrast, elevation of circulating adiponectin by either pharmacological or genetic intervention can decrease neointima formation and atherosclerosis in both rodents and rabbits. Adiponectin accumulates in the atherosclerotic lesion area, where it protects the vascular endothelium by promoting nitric oxide and alleviating oxidative stress, suppresses smooth muscle cell proliferation and migration, inhibits macrophage infiltration and foam cell formation, and ameliorates the collagen deposition. In line with these reports, our results demonstrated that adiponectin, but not FGF21, suppresses platelet-derived growth factor–induced proliferation and migration of smooth muscle cells and blocks...
LDL uptake and cholesterol accumulation in macrophages. On the other hand, the exacerbated smooth muscle proliferation and macrophage infiltration in the atherosclerotic plaques of DKO mice can largely be reversed by replenishment with adiponectin. Taken together, these findings suggest that the effects of FGF21 on smooth muscle cells and macrophages in the vessel walls are indirect, mediated in part by the induction of adiponectin.

Dyslipidemia, especially elevated LDL cholesterol, is a major contributor to atherosclerotic plaque formation. The cholesterol-lowering drugs, such as statins, have been used clinically to reduce the risk of coronary heart disease. Therapeutic administration of FGF21 has been shown to alleviate dyslipidemia in rodents,2 obese monkeys,22 and patients with type 2 diabetes mellitus,26 including reductions in total and LDL cholesterol and triglycerides, elevations in high-density lipoprotein cholesterol, and a shift to a less atherogenic apolipoprotein profile. Consistent with these pharmacological studies, our present study showed that FGF21 deficiency in apoE–/– mice causes a further aggravation of hypercholesterolemia and a shift of apolipoprotein profiles from high-density lipoprotein to LDL. Notably, the severe hypercholesterolemia in DKO mice is accompanied by augmented de novo cholesterol biosynthesis and increased expression of several cholesterologenic genes in the liver, suggesting that endogenous FGF21 is a physiological suppressor of hepatic cholesterol production. However, whereas adiponectin replenishment reverses hypertriglyceridemia, it has little effect on hypercholesterolemia and augmented hepatic cholesterogenesis in DKO mice, suggesting that the cholesterol-lowering activity of FGF21 is independent of adiponectin. Given that hepatic FGF21 expression is progressively elevated with the development of hypercholesterolemia in apoE–/– mice, it is possible that FGF21 acts as a sensor of cholesterol overload, which in turn prevents further worsening of hypercholesterolemia via its autocrine inhibition of hepatic cholesterogenesis.

Srebps, which structurally belong to the basic helix-loop-helix-leucine zipper transcription factor family, are the principal regulator of lipid synthesis.29 Unlike other members of this class of transcription factor, Srebps are synthesized as membrane-bound precursors that require cleavage by a 2-step proteolytic process to release their amino-terminal transactivation domain into the nucleus to bind to a specific DNA

**Figure 7.** Effects of fibroblast growth factor (FGF) 21 and adiponectin (ADN) on several key transcription factors involved in cholesterol metabolism. The liver samples from apolipoprotein (apo) E–/– mice or apoE–/– FGF21–/– (DKO) mice treated with recombinant mouse FGF21, ADN, or vehicle as in Figure 4 were subjected to real-time polymerase chain reaction or Western blot analysis. A and B, The relative mRNA and protein expression levels of liver X receptor (LXR) α, farnesoid X receptor (FXR), and sterol regulatory element-binding protein (Srebp)-1. C and D, The relative mRNA and protein expression of Srebp-2. E and F, The DNA binding activities of Srebp-1 and Srebp-2 in the nuclear extracts of liver tissues. n=5 to 7. Data are presented as dot plots with the line indicating the median. The global significance among 4 groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.
sequence (sterol regulatory element) and activate their target genes.29 Hepatic expression and activity of Srebps are tightly regulated at both transcriptional and posttranslational levels by metabolic hormones and nutritional factors.29 Srebp-1a and 1c preferably activate transcription of genes involved in fatty acid synthesis, whereas Srebp-2 displays strong specificity for genes involved in cholesterol biosynthesis.39,40 Our present study demonstrated that the expression and transcriptional activity of Srebp-2, but not Srebp-1, is significantly enhanced by FGF21 deficiency but is markedly suppressed by FGF21 treatment. Furthermore, adenovirus-mediated silencing of hepatic Srebp-2 expression is sufficient to counteract exacerbation of hypercholesterolemia and augmentation of hepatic cholesterol biosynthesis caused by FGF21 deficiency, whereas the therapeutic benefits of systemic FGF21 administration on the inhibition of hepatic cholesterogenesis and reduction of hypercholesterolemia are abrogated by overexpression of Srebp-2. Thus, our study identifies hepatic Srebp-2 as a key intracellular mediator conferring the regulatory effects of FGF21 on cholesterol homeostasis.

Although the precise signaling pathways whereby FGF21 selectively suppresses hepatic Srebp-2 remain unclear,
differential regulation of Srebp-1 and Srebp-2 has been reported in several previous studies. A high-carbohydrate diet induces the mRNA and protein expression of Srebp-1 but not Srebp-2, whereas dietary cholesterol enhances the expression of Srebp-2 and Srebp-1c but not Srebp-1a. Notably, FGF21 has been shown to form a regulatory loop with Sirt1 to reduce the treatment of atherosclerosis, instead of diabetes mellitus. Additional investigation is warranted to interrogate the role of the sirtuin family members in mediating FGF21-induced suppression of hepatic Srebp-2.

There are several limitations in our study. First, our observations are solely based on rodent models. In light of the fact that there is a difference in lipid metabolism and cardiovascular structure between rodents and humans, the pathophysiological relevance of our findings remains to be confirmed in humanoid large animals (eg, pigs) and in clinical studies. Second, although our data demonstrated the obligatory role of β-klotho and FGFR2 in mediating the cholesterol-lowering effects of FGF21 via suppression of Srebp-2 in the liver, the signaling pathways that link the FGF21 receptor with its regulation of cholesterol metabolism need further investigation.

In summary, our present study uncovers the protective effects of FGF21 against atherosclerosis via the induction of adiponectin in adipose tissue, reduction of hypercholesterolemia by suppression of hepatic Srebp-2, and augmentation of cholesterol efflux possibly by increasing ABCG5/8 expression. The results of our study highlight the potential of FGF21 in mediating FGF21-induced suppression of hepatic Srebp-2. In conclusion, the importance of atherosclerosis and cardiovascular disease remains to be confirmed in clinical trials.

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References
Atherosclerosis is a chronic inflammatory disease with many risk factors, including obesity, insulin resistance, diabetes mellitus, and dyslipidemia. Therefore, the therapeutic interventions targeting a single risk factor (eg, the use of statins to decrease hypercholesterolemia) are often insufficient to block the progression of atherosclerotic disease. In this study, we demonstrated that the liver-secreted hormone fibroblast growth factor (FGF) 21 potently alleviates atherosclerotic plaque formation and decreases premature death in apolipoprotein E–/– mice via several mechanisms. First, FGF21 induces the expression and increases premature death in apolipoprotein E–/– mice via several mechanisms. First, FGF21 induces the expression and decreases premature death in apolipoprotein E–/– mice via several mechanisms. First, FGF21 induces the expression of several transcripts in aortic tissues, including those for adenosine monophosphate-activated protein kinase (AMPK), which is known to regulate fatty acid metabolism and glucose transport. Second, FGF21 acts in the liver to decrease hepatic triglyceride and cholesterol synthesis. Third, FGF21 regulates the expression of several genes involved in lipid metabolism in hepatocytes, including those for sterol regulatory element-binding protein (SREBP) 1a and 1c. Finally, FGF21 reduces the expression of inflammatory cytokines in aortic tissues, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are known to contribute to the development of atherosclerosis.

To further validate the anti-atherosclerotic effect of FGF21, we used a murine model of diet-induced obesity and compared the effects of FGF21 treatment with those of a control group. Our results showed that FGF21 treatment led to a significant reduction in atherosclerotic plaque area in the aorta and reduced the expression of several pro-inflammatory cytokines in aortic tissues. These findings suggest that FGF21 may have potential therapeutic applications in the treatment of atherosclerosis and other inflammatory diseases.

In conclusion, our study demonstrates that FGF21 is a promising therapeutic target for the management of atherosclerosis and other inflammatory diseases. Further studies are needed to investigate the mechanisms by which FGF21 acts in the liver and to evaluate its effects in human subjects.
SUPPLEMENTAL MATERIAL

Supplementary methods

Mice

FGF21 knockout mice in C57BL/6J background were generated as previously described\(^1\). C57BL/6J apoE\(^{-/-}\) mice were obtained from Jackson Laboratory. ApoE\(^{-/-}\) mice were backcrossed with FGF21\(^{+/+}\) mice for at least ten generations to obtain apoE\(^{-/-}\) / FGF21\(^{+/+}\) mice (namely DKO mice). β-klotho-floxed mice were generated by Shanghai Nanfang Center for Model Organisms, and were used to produce β-klotho liver specific knockout mice (β-klotho LKO) by injecting with adeno-associated virus encoding Cre recombinase (AAV-Cre) under the control of the mouse apoE gene promoter. All the mice were housed in a room at controlled temperature [23±1°C] with a 12 hour light-dark cycle, and had free access to water and standard rodent diet. For the intervention studies, 8-week-old DKO and β-klotho LKO mice were treated with recombinant full-length adiponectin produced from mammalian cells\(^2\) and recombinant mouse FGF21\(^3\) by daily intraperitoneal injection respectively. Glucose and insulin tolerance tests were performed as described previously\(^4\). All the animal studies were approved by the animal research ethics committee of Wenzhou Medical University and the University of Hong Kong.

Analysis of Atherosclerotic Lesions

Oil red O staining was used to assess the size of the atherosclerotic lesion as previously described\(^5,6\) with slight modifications. For en face analyses of lesions in the entire aorta, after perfusion, whole aorta was dissected out, opened longitudinally from heart to the iliac arteries, pinned on a black wax pan, and stained with Oil red O. The images of the aorta were captured using a SONY DXC-970MD color video camera, analyzed with the Image-Pro plus program.
For analysis of plaque lesion in aortic sinus, the heart and proximal aorta were removed and embedded in optimum cutting temperature compound. Serial 10μm-thick cryosections from the middle portion of the ventricle to the aortic arch were collected. For analysis of lesion in brachiocephalic arteries, serial 10μm-thick cryosections from a 150μm-distance position to aortic arch were collected. Then, cryosections of aortic sinus and brachiocephalic arteries were stained with Oil Red O and hematoxylin. The lipid-containing area on each section was determined in a blinded fashion, using an ocular piece with a 20×20 μm² grid on a light microscope. The average lesion area per aorta, calculated from 5 to 10 sections of each aorta, was determined.

**Immunocytochemistry**

The cryosections of mouse aortic arches were fixed in acetone and blocked with non-immune rabbit serum, followed by incubation with a rabbit polyclonal antibody against the macrophage marker F4/80 (Abcam, Cambridge, MA) or smooth muscle α-actin (Sigma, St Louis, MO) for 90 minutes. After washing, the sections were incubated with a FITC- or Cy3-labeled secondary goat anti-rabbit antibody for 30min. All the slides were examined under the Olympus biological microscope BX41, and the images were recaptured with an Olympus DP72 color digital camera. Planimetry on the photographed cross-sections was performed using Image-Pro Plus version 5.0.1 (Media Cybernetics, Inc, Bethesda, MD). The lesion areas were defined as an area between the lumen and internal elastic lamina, and the cell contents were presented as the percentage of positive surface areas in the lesion area.

**Measurement of Cholesterol/cholesteryl Ester Levels in the Brachiocephalic arteries**

The analysis of cholesterol/cholesteryl ester abundance was measured with a commercial kit according to the manufacturer instruction (abcam 65359, Abcam, Cambridge, UK). Briefly, the brachiocephalic artery was dissected free of adventitial tissue, weighed, extracted with 200μl of chloroform: Isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer after weighting, and centrifuged at 15,000 x g for 10 minutes. The organic phase was transferred to a new
tube and air-dried at 50°C followed by vacuum drying for 30 min to remove trace organic solvent. The lipids were then dissolved with 200μl of Cholesterol Assay Buffer. 50ul of the samples or standard cholesterol were incubated with the reaction mixture including cholesterol esterase, cholesterol enzyme mix and cholesterol probe for 60 minutes at 37°C, followed by measurement of absorbance at 570nm with a microplate reader. Data were expressed as μmole cholesterol/cholesteryl ester per g tissue.

**Biochemical and Immunological Assays**

Plasma lipid profiles including total triglycerides, total-, HDL- and LDL-cholesterol levels were measured with commercial kits from Sigma, (St. Louis, MO). Liver total lipids were extracted with acetone and chloroform/methanol (2/1, v/v) as described\(^7\), and total cholesterol levels in the liver were quantified as above and expressed as mg cholesterol per gram liver tissue (wet). Plasma levels of insulin and total adiponectin were measured using immunoassays from Antibody and Immunoassay Services at the University of Hong Kong. MCP-1 and TNF-α concentrations were analyzed with immunoassays from R&D System Inc (Minneapolis, MN).

**Measurement of Cholesterol Absorption**

Intestinal cholesterol absorption was determined by a fecal dual-isotope ratio method\(^8\). Briefly, mice were injected with 2.5 μCi \(^3\)H-cholesterol in Intralipid (Sigma, St Louis, MO, USA) via tail vein, followed by oral gavage of 1 μCi \(^14\)C-cholesterol in median-chain triglycerides (MCT oil, Mead Johnson, Evansville, IN). Mice were returned to cage with free access to food and water. After 72 h, blood samples were collected and the radioactivity of \(^14\)C and \(^3\)H were determined by scintillation counting. Intestine cholesterol absorption was determined as the ratio of \(^{14}\)C/\(^3\)H in 1 ml of plasma.

**Hepatic de novo Cholesterol synthesis**

Hepatic cholesterol synthesis was determined as previous described\(^9\). Mice fasted for 4 hours were intraperitoneally injected with 10 μCi of [1-\(^14\)C]-sodium acetate (Perkin Elmer, Waltham, MA), and were then sacrificed at 30min after injection to harvest liver tissue. Approximately 250mg of liver tissue was rinsed in ice-cold PBS, and then saponified in 2.2
ml mixture of 50% KOH: 95% ETOH (1:10, v: v) at 70°C overnight. $^3$H-cholesterol (1 μCi) was added to the same tube as a recovery control. Sterols were extracted with 3 ml hexane, dried and redissolved in 300μl mixture of acetone : ETOH (1:1, v: v), followed by precipitation with 1 ml of digitonin (0.5% in 95% ETOH) overnight at room temperature. The radioactivity of $^3$H and $^{14}$C in the precipitates was determined in a scintillation counter. Cholesterol synthesis rate was expressed as the amount of [1-$^{14}$C]-acetate incorporated into sterols per minute per gram liver tissue.

**Measurement of Fecal Cholesterol and Bile Acids**

Feces were collected from individually housed mice over a 3-day period and were dried, weighed, and ground to a powder. Lipids were extracted from feces with chloroform/methanol (2:1), dried, and dissolved with 5% Triton X-100 in isopropanol. Cholesterol contents were quantified using a biochemical assay. For analysis of bile acids, 50 mg of dried feces were added to 2.2 ml ethanolic NaOH (0.08M) and heated (95°C) for 2 h. After cooling, neutral sterols were extracted three times with 5 ml hexane, followed by acidification with 2.5 ml of 0.16M HCl and extraction with 5 ml of ethyl acetate. The dried extract was solubilized in 1.25% Triton X-100 in 20% methanol. Bile acids in each sample were determined with a Bile Acid Assay Kit (Genzyme Diagnostic, Framingham, MA).

**RNA Extraction and Real-Time PCR**

Total RNA was extracted from liver or adipose tissues with TRIzol reagent (Invitrogen), and complementary DNA was synthesized from 0.5μg total RNA by reverse transcription with an ImProm-II reverse transcription kit (Promega) with random hexamer primers. Quantitative real-time PCR was performed on the Applied Biosystems Prism 7000 sequence detection system, with specific primers described in [Supplementary Table 1](#). The amplification efficiency, as calculated from the slope of each standard curve using the formula $E=10^{(-1/Slope)} \times 100$, is between 92 and 105%. The relative expression level of each gene was calculated with the Pfaffl methods as previously described$^{10}$, using the β-actin gene as the reference control for normalization.
Srebp-1 and Srebp-2 DNA Binding Activity Assay

Srebp-1 and -2 DNA binding activities were measured by a commercial kit according to the manufacturer instruction (ab133125 and ab133111, Abcam Cambridge, UK). In brief, approximately 10mg nuclear extracts isolated from liver tissue were added to 96-well microplates coated with a specific double strand DNA (dsDNA) sequence containing with Srebp-1 or -2. The unbound reagents were removed by extensive washing, followed by sequential incubation with a primary antibody against Srebp-1 or -2 for 2 h, and a HRP-conjugated secondary antibody was added and incubated for 30 minutes. After washing, the substrate reagents were added, and the absorbance values at 450nm were measured with a microplate reader.

Construction of Adenoviral Vectors for Knockdown Srebp-2, FGFR2, FGFR3 and FGFR4 or Overexpression of Srebp-2

An adenovirus delivery system was used for knocking down Srebp-2, FGFR2, FGFR3 and FGFR4 expression with small hairpin RNA as well as for overexpression of Srebp-2. The oligonucleotides for generation of siRNA against these genes as well as the corresponding scrambled controls are listed in [Supplementary Table 2](#). The forward and reverse oligonucleotides were annealed, ligated into pENTR/U6 entry vector, and then subcloned into pAd/BLOCK-iT DEST vector through recombination. To construct adenoviral vectors for overexpression of Srebp-2, cDNA encoding amino acids 1–460 of mouse Srebp-2 was inserted into pshuttle-CMV vector, and then subcloned into pAdeasy-1 adenoviral backbone vector (Stratagene) through recombination in Escherichia coli.

To package adenovirus, the adenoviral vectors were linearized with the restriction enzyme PacI and transfected into HEK293 cells using Lipofectamine 2000. After several rounds of propagation, recombinant adenovirus was purified by an AdEasy virus purification kit (Stratagene), and the titer was determined with an endpoint assay as described11.

Smooth Muscle Proliferation and Migration Assays

Human aortic smooth muscle cells (HASMCs) were maintained in a humidified 37 °C and 5% CO₂ environment in OPTI-MEM® supplemented with 10% fetal bovine serum, 0.01 mg/ml
Supplemental materials_FGF21 and atherosclerosis by Lin Z.

insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, and 0.05 mg/ml ascorbic acid. Cells were routinely split at a 1:4 ratio, and cultures between the third to eighth passages were used. For proliferation analysis, cells were plated in 24-well plates (8000 and 10000 cells/well for HASMCs) and grown for 24 h. Medium was then replaced with DMEM with 0.1% BSA for 24 h. The medium was subsequently changed to fresh DMEM with 1% fetal bovine serum plus PDGF-BB (10ng/ml), recombinant mouse FGF21 (50ng/ml) and/or recombinant mouse adiponectin (2.0μg/ml). 18 h after incubation, 1 μCi of [methyl-3H] thymidine was added into each well for another 6 h, and the amount of [3H] thymidine incorporated into DNA in each well was determined as we described previously12.

To assess cell migration, a Boyden chamber assay was performed using Transwell chambers (6.5mm, model 3422; Costar, Cambridge, MA, USA) with an 8-mm pore polycarbonate membrane13. Growth-arrested HASMC were harvested, suspended in serum-free DMEM. Cells were added to the upper chamber of the Transwell at 5×10^5 cells in 100 μl/well. A total of 600 μl of serum-free DMEM was added to the lower chamber, followed by treatment with PDGF-BB(10ng/ml), recombinant full-length adiponectin (2.0μg/ml) or recombinant mouse FGF21(50ng/ml) for 24 h respectively. Afterwards, the nonmigratory cells were removed from the upper surface of the membrane by scraping with cotton swabs. Membrane was then fixed with methanol, stained with Diff-Quik solution (Baxter, McGaw Park, IL, USA) and mounted on a glass slide. Migrated cells were counted at x400 magnification in 10 microscope fields per filter.

**Analysis of Macrophage Uptake of Acetylated LDL (AcLDL)**

AcLDL labeled with the fluorescent probe 3,3,39,39-tetramethylindocarbocyanine perchlorate (Dil-AcLDL) and unlabeled AcLDL were purchased from Biomedical Technologies (Ward Hill, MA 01835 USA). Primary mouse peritoneal macrophages grown in a serum-free RPMI-1640 medium were treated with mouse adiponectin (2.0μg/ml) or rmFGF21(50ng/ml) for 24 h, and were then incubated with 10μg/ml Dil-AcLDL dissolved in RPMI-1640 containing 2% lipoprotein-deficient serum (Sigma, St. Louis, Missouri, USA) for 3 hours. For competition assays, unlabeled AcLDL in excess amounts (50-fold) were added
together with Dil-AcLDL. The cells were resuspended in PBS and analyzed using a FACScan flow cytometer (Becton Dickinson). Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from the mean fluorescent intensity of Dil-labeled cells. The uptake of Dil-AcLDL was calculated by subtracting binding in presence of excess amount of unlabeled AcLDL from total binding in absence of unlabeled acLDL, and expressed as fold changes over untreated cells.

**Generation of β-klotho Liver specific knockout mice**

C57BL/6J mice with the β-klotho gene floxed were generated by Shanghai Nanfang Center for Model Organisms. The targeting construct containing the two loxP sites flanking the exon 2 of the β-klotho gene and the FRT-flanked neomycin selection cassette (supplementary Fig.7A) was electroporated into embryonic stem cells, followed by selection of positive ES clones, microinjection and chimera identification as described previously. Chimeric males were mated with C57BL/6J females for at least 8 generations to produce homologous β-klotho-floxed mice (β-klotho<sup>f/f</sup>) in C57BL/6 background. Genotypes of mice were determined by PCR using the following primers: P1 (5'- TGTTGGGCCATCTAAAATGG-3'), P2 (5'- GCCAAGACAAACATATTCGGG-3'), which produces a 216-bp fragment for the β-klotho-floxed locus, and a 132-bp fragment for wild-type (WT) locus respectively.

To generate β-klotho liver-specific knockout (Klb-LKO) mice, 1 × 10<sup>12</sup> genomic particles of adeno-associated virus encoding Cre recombinase (AAV-Cre) or green fluorescent protein (AAV-GFP, as wild-type control) under the control of the mouse apoE gene promoter (which drives hepatocyte-specific gene expression) were intravenously injected into β-klotho<sup>f/f</sup> mice as previously reported. After 4 weeks of AAV injection, mice were sacrificed to obtain various tissues for Western blot analysis to obtain liver-specific deletion of β-klotho.

**References**


2. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin
alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J Clin Invest.* 2003;112:91-100


**Supplementary Table 1**: A list of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse primer 5’ to 3’</th>
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<tr>
<td>Mouse adiponectin</td>
<td>AGACCTGGCCACCTTTCTCTCATT</td>
<td>AGAGGAAACAGGAGAGCTTGCAACA</td>
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<td>Mouse CYP7A1</td>
<td>CTGTCATACCACAAAGTTCTTATGCA</td>
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<td>Mouse TNF-α</td>
<td>TCCTCTAGACACACCCAACCT</td>
<td>GAGGCCCAGTTTAGACTT</td>
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<td>Mouse β-actin</td>
<td>GGGCTATTTCCCTCCTCAGT</td>
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**Supplementary Table 2**: The oligonucleotides used for construction of adenoviral siRNA for Srebp-2, FGFR2, FGFR3, FGFR4 and scrambled control.

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<td>Sh-scramble (forward)</td>
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<td>Sh-scramble (reverse)</td>
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<tr>
<td>Sh-FGFR4(forward)</td>
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<td>Sh-FGFR4(reverse)</td>
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<td>Sh-Scramble (reverse)</td>
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<td>Sh-FGFR2(forward)</td>
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<td>Sh-FGFR2(reverse)</td>
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<td>Sh-Scramble (forward)</td>
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<td>Sh-Scramble (reverse)</td>
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<td>Sh-Scramble (forward)</td>
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<tr>
<td>Sh-Scramble (reverse)</td>
<td>5'-AAAAAACGAGACAGACGAGUCCAGGAATGCTGTGGGCTCTTCATAATATT-3'</td>
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Supplemental Figure 1. Dynamic changes in circulating levels and hepatic mRNA expression of FGF21 in apoE⁻/⁻ mice and C57BL/6J mice. The tissue samples were collected at 4-, 12- and 24-week-old male mice. (A) Serum levels of FGF21 measured with an immunoassay. (B) The mRNA expression level of FGF21 in the liver as determined by real-time PCR. n=6. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed using repeated measure ANOVA.
Supplemental Figure 2. Genotyping of FGF21−/−apoE−/− (DKO) mice. FGF21 KO mice were generated by replacing most of exon 1 and all of exons 2 and 3 of the FGF21 gene with the IRES-LacZ-polyA/PKG-neo cassette. Genotypes of mice were determined by PCR using the following primers: P1-Fgf21 (5’-GACTGTTCAGTCAGGGATTG-3’), P2-Fgf21 (5’-CCCGTGATATTGCTGAAGAG-3’), and P3-Fgf21 (5’-ACAGGGTCTCAGGTTCAA AG-3’). P1 and P3 produced a 541-bp fragment of the wild-type (WT) Fgf21 locus. P2 and P3 produced a 243-bp fragment of a mutant Fgf21 locus. Genotypes of apoE−/− mice were determined by PCR using the following primers: P1-apoE (5’-TAT CTA AAC AGACTC CACAGCTCCAGACC-3’), P2-apoE (5’-GACTGGGCACA ACA GAC AAT CGG CTG CTCT-3’), P3-apoE (5’-CGAAGCCAGCTTGAGTTACAGAATG GGATC -3’). P1 and P3 produced a 400-bp fragment of the wild-type apoE locus. P2 and P3 produced a 600-bp fragment of a mutant apoE locus. (A) Representative PCR genotyping results. (B) Confirmation of FGF21 protein deficiency in the liver tissue of DKO mice by immunoblotting analysis.
Supplemental Figure 3. FGF21 deficiency does not influence body weight, glucose tolerance and insulin sensitivity in apoE−/− mice. DKO and apoE−/− mice on standard chow were monitored for (A) body weight, (B) glucose and insulin levels, (C,D) Glucose and insulin tolerance tests were performed at 19 and 20 weeks after birth respectively. n=6. Data are presented as mean ± SEM (A, C and D) or dot plots with the line indicating the median (B).
**Supplemental Figure 4.** FGF21 deficiency exacerbates HFHC diet-induced atherosclerosis, hyperlipidemia and inflammation. 8-week-old apoE<sup>−/−</sup> mice or DKO mice were fed with HFHC diet for a period of 12 weeks. STC-fed apoE<sup>−/−</sup> mice were included as a control. (A) En face staining of entire aortas with Oil red O. (B) Quantification of aortic plaque areas. (C-G) Serum lipid profiles, including triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL). (H,I) Serum levels of MCP-1 and TNF-α. n = 6-8. ***, p<0.001 vs. vehicle. Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with a Dunn-Sidak procedure.
Supplemental Figure 5. Circulating levels of FGF21 and adiponectin in DKO mice after intraperitoneal administration of recombinant mouse FGF21 or adiponectin. Serum samples were collected every hour until 6 hours after DKO mice were intraperitoneally injected with FGF21 (0.1 mg/Kg.day), adiponectin (10 mg/Kg.day) or PBS as vehicle control. (A-B) Serum levels of FGF21 and adiponectin at different time points after injection with FGF21. (C) Changes in serum levels of adiponectin after administration with recombinant mouse adiponectin. Note that serum FGF21 is not detectable in DKO mice. n = 5. ***, p<0.001 vs. vehicle. Data are presented as mean ± SEM. Repeated measure ANOVA was used for comparison between two groups at each time points.
Supplemental Figure 6. Adiponectin, but not FGF21, directly suppresses smooth muscle cell proliferation and migration, and LDL uptake of macrophages. (A,B) Human aortic smooth muscle cells were treated with PDGF-AA (0.1μg/ml), adiponectin (ADN, 2.0μg/ml) or recombinant mouse FGF21 (50ng/ml) for 24 hours. The cell proliferation (A) and migration (B) was determined by ³H-thymidine incorporation assay and a modified Boyden chamber assay described in respectively. (C) Primary mouse peritoneal macrophages were treated with ADN (2.0μg/ml), recombinant mouse FGF21 (50ng/ml) or vehicle (PBS) for 24 hours, loaded with Dil-AcLDL (10μg/ml). The uptake of Dil-AcLDL was determined by flow cytometry as described in the method, and expressed as fold changes over untreated cells. n = 5. Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.
A) Schematic diagram of the genetic modifications:

- WT: Normal wild type
- β-klotho: Gene of interest
- β-actin: Reference gene
- Klb-LKO: Knockout allele
- Klb-floxed: Floxed allele
- Exon: Exons of the gene
- AAV-Cre: Cre recombinase

B) Western blot analysis showing protein expression:

- Liver
  - β-klotho
  - β-actin

- WAT
  - β-klotho
  - β-actin

C) Experimental timeline:

- Week 8
  - Klb-floxed C57BL/6J mice
  - HFHC diet
  - AAV-Cre / AAV-GFP

- Week 14
  - rmFGF21 (daily i.p. administration)

- Week 16
  - Sacrificing

D) Graph showing Stebp-2 mRNA levels:

- WT: Control group
- Klb-LKO: Knockout group
- FGF21: Treatment condition

E) Graph showing DNA binding activity:

- WT: Control group
- Klb-LKO: Knockout group
- FGF21: Treatment condition

F) Graph showing relative mRNA levels:

- HMGCR: HMG-CoA reductase
- Fdps: Fatty acid synthase
- HMGCS: HMG-CoA synthase

G) Graph showing cholesterol levels:

- WT: Control group
- Klb-LKO: Knockout group
- FGF21: Treatment condition

H) Graph showing adiponectin levels:

- WT: Control group
- Klb-LKO: Knockout group
- FGF21: Treatment condition

NS: Not significant
Supplemental Figure 7. The inhibitory effects of FGF21 on cholesterol biosynthesis are abrogated by liver-selective depletion of β-klotho. (A) Strategies for generating β-klotho liver-specific knockout mice (Klb-LKO) in C57BL/J background. 8-week-old β-klotho-floxed mice (Klb-floxed) were intravenously injected with $1 \times 10^{12}$ genomic particles of adeno-associated virus encoding Cre recombinase (AAV-Cre) to delete the exon-2 region floxed with the two loxp sites for generation of β-klotho-LKO, or AAV encoding GFP as a wild-type (WT) controls. (B) Western blot analysis to confirm the specific deletion of β-klotho in the liver tissue in β-klotho-LKO mice, after 4 weeks of injection with AAV encoding Cre recombinase. Note that β-klotho remains unchanged in epididymal white adipose tissue (WAT) of Klb-LKO mice. The deletion of β-klotho can sustain for over 30 weeks after injection with AAV-Cre recombinase (data not shown). (C) A schematic diagram showing the study design for panel D-H. (D) The relative expression levels of Srebp-2 in the liver. (E) The DNA binding activities of Srebp-2 in the nuclear extracts of liver tissues. (F) The relative mRNA expression levels of genes involved in cholesterol synthesis including HMGCR, HMGCS and Fdps. (G) Serum total cholesterol levels. (H) Circulating levels of adiponectin. NS, not significant. n = 7. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed with two-way ANOVA (D-H).
Supplemental Figure 8. Hepatic FGFR2 is involved in FGF21-mediated suppression of Srebp-2, expression of cholesterologenic genes and hypercholesterolemia. (A) The relative mRNA expression levels of the four FGFRs in the liver tissues of 12-week-old male C57BL/6J mice. (B-G) 10-week-old C57BL/6J mice on HFHC diet were intravenously injected with 1x10⁹ p.f.u of adenovirus encoding siRNA specific to FGFR2 (Ad-siR2) or scramble control (Ad-scramble) for 7 days, followed by daily intraperitoneal administration of rmFGF21 (0.1 mg/Kg) for another 7 days. (B, C) The relative mRNA and protein expression of FGFR2 in the liver at 14 days after the adenoviral administration. (D,E) The relative mRNA levels and DNA binding activities of Srebp-2 in the nuclear extracts of liver tissues. (F) The relative mRNA expression levels of genes involved in cholesterol synthesis including HMGCR, HMGCS and Fdps. (G) Serum total cholesterol (TC) levels. n = 6. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed with Mann-Whitney U test (B) or two-way ANOVA (D-G) to compare two groups or multiple groups respectively.
Supplemental Figure 9. The proposed mechanism whereby FGF21 suppresses atherosclerosis via induction of adiponectin (ADN) and suppression of Srebp-2.